

Please replace the two sheets of drawings containing Figures 1 and 2 filed with the application on January 23, 2004 with the attached two sheets of Formal Replacement Drawings containing Figures 1 and 2.

REMARKS

Amendments to the Specification. The brief description of Figure 1 starting on page 4, line 17 has been amended to include the information from the legend deleted herein from Figure 1 as filed. The remaining amendments to the specification correct grammatical and typographical errors.

Amendments to the Claims. With entry of this amendment, claims 2, 6-9, 17-26, 28, 30, 32, and 36 have been amended and claims 1, 4, 5, 10, 11, 16, 35, and 37-49 have been canceled. The Office Action inadvertently omits claim 36 from the list of pending claims and claim 37 from the list of withdrawn claims. *See* page 2.

Claim 2 has been amended to delete the phrase “or prevention,” add that the patient treated in accordance with the claimed method has thrombocythemia, add that the anagrelide or salt thereof is in a skin permeable form, replace “in a manner avoiding” with “to minimize,” and limits the presently claimed route of administration to transdermal administration. The foregoing amendments have been made without prejudice to the filing of an appropriate continuation application(s) directed to the non-elected forms of the invention. Each of these amendments has been made to more particularly point out and distinctly claim the invention set forth herein. Support for “a skin permeable form of” anagrelide or salt thereof can be found on, *e.g.*, page 5, lines 19-24 and page 10, lines 4-18. Support for “to minimize” first pass liver metabolism can be found on page 4, line 30 to page 5, line 4 and page 2, lines 27-29.

Claim 18 has been amended to add oleic acid to the list of recited skin permeation enhancers. Support for this amendment can be found on page 21, line 3 and page 30, line 14. The remaining claim amendments either amend the dependency of claims to reflect the cancellation of claims or correct antecedent basis in the claims.

Thus, claims 2, 3, 6-9, 12-15, 17-34, and 36 are pending and the subject of examination.

Amendment to the Drawings. With entry of this amendment, the two sheets of drawings filed January 23, 2004 have been replaced with two sheets of Formal Replacement Drawings. The Formal Replacement Drawings differ from the Drawings as originally filed in that the Replacement Drawings lack legends.

All amendments find support in the application as filed and, thus, no new matter has been added to the specification, claims, or drawings.

Objection to the Drawings

The Examiner has objected to Figure 1 because the presence of a legend in Figure 1 is improper. Applicant submits herewith Formal Replacement Drawings in which the legend of Figure 1 is deleted and respectfully request withdrawal of the objection.

Rejection under 35 U.S.C. §112, First Paragraph - Enablement

Claims 2-9, 12-15, 17-34, and 36 have been rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. According to the Examiner, claim 2 is enabled for the treatment of thrombocytopenia by transdermally administering an effective amount of anagrelide or a pharmaceutically acceptable anagrelide salt. However, the Examiner contends that the prevention of thrombocytopenia as claimed is not supported by the specification and would require undue experimentation because no examples of the prevention of thrombocytopenia are provided.

The claims have been amended to recite only the treatment and not the prevention of thrombocytopenia. Thus, this enablement rejection has been rendered moot and Applicant respectfully requests withdrawal of this rejection.

Rejection under 35 U.S.C. §102(b) – Anticipation

Claim 2 has been rejected as anticipated by U.S. Patent No. 6,156,753 (“Doherty”) in light of U.S. Patent No. 6,024,975 (“D’Angelo”). According to the Examiner, Doherty teaches the

transdermal administration of anagrelide. The Examiner asserts that treating thrombocytopenia using anagrelide is inherent. D'Angelo is used by the Examiner as a secondary reference to show that drugs administered transdermally as described by Doherty are not subject to liver metabolism.

Applicant has amended claim 2 to clarify that this claim sets forth a method to treat patients with thrombocytopenia. In light of this amendment, Applicant respectfully traverses the rejection. According to MPEP §2112.02, a new use of an old structure based on unknown properties of the structure may be patentable if the use is not a result or property of the known composition. *See also* Perricone v. Medicis Pharmaceutical Corp., 432 F. 3d 1368 (Fed. Cir. 2005). Doherty describes treating erectile dysfunction by administering anagrelide transdermally (*see* claims 1 and 3). Doherty does not describe or suggest treating thrombocytopenia by administering anagrelide transdermally as set forth in claim 2. The treatment of thrombocytopenia is a new use of transdermally administered anagrelide not an inherent result or property of the composition and use disclosed by Doherty. In other words, thrombocytopenia is not inherently treated when anagrelide is transdermally administered to treat erectile dysfunction because thrombocytopenia and erectile dysfunction are different afflictions with separate patient populations. Thus, since the transdermal administration of anagrelide to treat thrombocytopenia is a new, non-inherent use of anagrelide as set forth in claim 2, claim 2 is novel and Applicant respectfully requests withdrawal of the anticipation rejection.

First Rejection under 35 U.S.C. §103 – Obviousness

Claims 2-9, 12, 17-20, 26, 27, 32, and 36 have been rejected as obvious over U.S. Patent No. 6,194,420 ("Lang") in view of U.S. Patent No. 6,221,383 ("Miranda") and in further view of D'Angelo. The Examiner asserts that Lang teaches anagrelide containing pharmaceutical compositions to treat essential thrombocytopenia. According to the Examiner, Miranda describes the transdermal administration of anagrelide. The Examiner asserts that D'Angelo teaches the transdermal delivery of drugs using a patch system. The Examiner contends that one of ordinary skill in the art would be motivated to combine the teachings of Lang with the teachings of Miranda because both describe the administration of anagrelide as part of a pharmaceutical composition.

The Examiner further asserts that one skilled in the art would combine the teachings of Lang, Miranda, and D'Angelo because when the teachings of Lang and Miranda are combined, these teachings overlap with D'Angelo in subject matter, *i.e.*, the administration of medicaments, particularly anagrelide, by transdermal delivery.

Second Rejection under 35 U.S.C. §103 – Obviousness

Claims 21-23 and 28-31 have been rejected as obvious over Lang in view of D'Angelo and in further view of U.S. Patent No. 5,133,972 (“Ferrini”). The Examiner incorporates by reference the discussion of Lang and D'Angelo from the above rejections. Ferrini, according to the Examiner, teaches a multilayered therapeutic system for the transdermal administration of an active ingredient. The Examiner asserts that a skilled artisan would be motivated to combine Lang, D'Angelo, and Ferrini because each describes the administration of medicaments by transdermal delivery.

Third Rejection under 35 U.S.C. §103 – Obviousness

Claims 13-15, 24, 25, 33, 34, and 36 have been rejected as obvious over Lang in view of D'Angelo and in further view of U.S. Patent No. 4,847,276 (“Yarrington”). The Examiner incorporates by reference the discussion of Lang and D'Angelo from the above rejections. The Examiner asserts that Yarrington teaches the treatment of thrombocythemia by the administration of anagrelide using particular regimens. According to the Examiner, one of ordinary skill in the art would be motivated to combine the teachings of Lang, D'Angelo, and Yarrington because each relate to the treatment of thrombocythemia.

Applicant's Response to the Obviousness Rejections

Applicant respectfully traverses each of the obviousness rejections.

Lang primarily discloses the use of the anagrelide metabolite, 2-amino-5,6-dichloro-3,4-dihydroquinazoline, to treat thrombocythemia. In fact, subsequent published research has shown

this metabolite to be entirely without activity in reducing blood platelets (Wang *et al.*, *British Journal of Pharmacology* 2005, 146:324-332; provided herewith). Lang also briefly describes routes of administration, including capsule, tablet, enteric coated tablet, IV formulation, nasal spray, and intraperitoneal injection, of the metabolite (*see* column 6, lines 41-50 and column 11, lines 39-43). Although Lang describes the use of anagrelide to treat thrombocythemia, no routes of administration of anagrelide are disclosed or suggested.

Miranda describes a transdermal drug delivery system. Anagrelide is mentioned a single time as one of over a dozen antithrombotic drugs that can be administered using this system and is among a laundry list of drugs extending from column 11, line 61 to column 32, line 42. Thrombosis and thrombocythemia are distinct conditions. Thrombosis is the formation of blood clots whereas thrombocythemia is a disorder associated with the increased or abnormal production of blood platelets. Although blood clotting can result from platelet aggregation, patients with thrombosis do not necessarily have thrombocythemia and patients suffering from thrombocythemia do not necessarily have thrombosis. Furthermore, not all antithrombotic drugs can be used to treat thrombocythemia. For example, Miranda lists argatroban and defibrotide as antithrombotic drugs in addition to anagrelide (*see* column 25, lines 56-59). Argatroban directly inhibits thrombin and defibrotide inhibits platelet aggregation. Neither of these drugs can treat thrombocythemia because neither drug reduces platelet production. Thus, the use of anagrelide to treat thrombocythemia is not disclosed or suggested in Miranda.

D'Angelo describes a transdermal drug delivery system, but neither describes nor suggests the administration of anagrelide. D'Angelo also does not describe or suggest the treatment or prevention of thrombocythemia.

Ferrini describes the topical administration, including the transdermal administration, of methanediphosphonic acid derivatives. Ferrini does not describe or suggest anagrelide or the treatment of thrombocythemia.

Yarrington primarily teaches the treatment of thrombocytosis by administering 5-(4-chlorophenyl)-2,4-dimethyl-3H-1,2,4-triazole-3-thione. Yarrington discloses that 5-(4-chlorophenyl)-2,4-dimethyl-3H-1,2,4-triazole-3-thione can be administered orally or parentally including by implant or injection (*see, e.g.*, column 3, line 33 to column 4, line 5). Anagrelide is briefly discussed in the background of the invention as a compound used to treat thrombocytosis (*i.e.*, thrombocythemia) (*see* column 2, lines 2-17) without any discussion or suggestion of the mode of its administration.

Thus, the cited references taken separately or in combination do not describe or suggest the treatment of thrombocythemia in patients afflicted by that disease by transdermally administering an effective amount of a skin permeable form of anagrelide to minimize first pass liver metabolism as recited in the present claims. Furthermore, the present claims would not have been obvious to one of ordinary skill in the art because Applicant unexpectedly discovered that transdermally administering anagrelide to minimize first pass liver metabolism as set forth in the claims would circumvent the adverse cardiovascular side-effects observed when anagrelide is administered orally. This surprising result is discussed in the Declaration pursuant to 37 C.F.R. §1.132 by Dr. Richard Franklin (“the Franklin Declaration”), the present inventor, submitted herewith.

Dr. Franklin in his Declaration explains that adverse cardiovascular side-effects plague many thrombocythemia patients who are orally treated with anagrelide (§ 7 of the Franklin Declaration). Dr. Franklin surprisingly discovered that these side-effects were caused by the 3-hydroxy metabolite of anagrelide formed during its first pass liver metabolism (§ 7 of the Franklin Declaration). 3-Hydroxy anagrelide showed a remarkable 40-fold increase in inhibitory activity to phosphodiesterase III (PDEIII), an enzyme known to regulate cardiovascular functions, compared to anagrelide (§ 7 of the Franklin Declaration). As a result, patients who were orally administered anagrelide experienced negative side-effects after anagrelide was extensively metabolized to the 3-hydroxy metabolite, an exceedingly potent inhibitor of PDEIII (§ 7 of the Franklin Declaration).

Request for Rejoinder of Method and Composition Claims

- the method for reducing the platelet count in a patient with thrombocythemia (Claim Group II (canceled claim 37) of the Restriction Requirement mailed December 19, 2006) and
- the method for reducing the side effects associated with the oral administration of anagrelide (Claim Group III (canceled claim 38) of the Restriction Requirement mailed December 19, 2006)

using the same method as recited in claim 2. The method of treating thrombocythemia by transdermal administration of anagrelide in claim 2 encompasses reducing the platelet count in a patient with thrombocythemia since administering anagrelide is known to reduce platelet counts. The method recited in claim 2 also encompasses the method for reducing the side effects associated with the oral administration of anagrelide since transdermally administering anagrelide avoids the

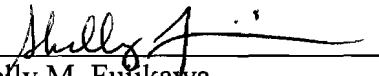
conversion of anagrelide into its 3-hydroxy metabolite thereby reducing the side effects associated with the oral administration of anagrelide.

Conclusion

In view of the above remarks, it is respectfully requested that the application be reconsidered, all pending claims be allowed and the case be passed to issue. If there are any other issues remaining which the Examiner believes could be resolved through a Supplemental Response or an Examiner's Amendment, the Examiner is respectfully requested to contact the undersigned at the telephone number indicated below.

Dated: September 19, 2007

Respectfully submitted,

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Comparison of the biological activities of anagrelide and its major metabolites in haematopoietic cell cultures

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1 The platelet-lowering drug anagrelide inhibits bone marrow megakaryocytopoiesis by an unknown mechanism. Recently, it was found that anagrelide is bio-transformed in humans into two major metabolites (6,7-dichloro-3-hydroxy-1,5 dihydro-imidazo[2,1-b]quinazolin-2-one (BCH24426) and 2-amino-5,6-dichloro-3,4,-dihydroquinazoline (RL603). Whether these metabolites have biological activities that may underlie the mode of action of the parent drug is presently unclear. To clarify this question here we have compared the activities of anagrelide, BCH24426 and RL603 on the growth and differentiation of CD34⁺ haematopoietic progenitor cells in liquid culture and on the migration of differentiated megakaryocytes.

2 Incubation with either anagrelide, BCH24426 or RL603 did not affect the early expansion of CD34⁺ cells stimulated by thrombopoietin. In contrast, both anagrelide and BCH24426 potently inhibited the development of megakaryocytes ($IC_{50} \pm s.e.m. = 26 \pm 4$ and 44 ± 6 nM, respectively), whereas RL603 showed no significant effect.

3 Anagrelide and BCH24426 did not affect erythroid or myelomonocytic differentiation stimulated by erythropoietin or granulocyte-macrophage colony-stimulating factor, demonstrating the selectivity of these compounds against the megakaryocytic lineage.

4 Neither anagrelide nor its metabolites showed a significant effect on the migratory response of megakaryocytes towards stromal cell-derived factor-1 α .

5 Although BCH24426 was shown to be considerably more potent than anagrelide as an inhibitor of phosphodiesterase type III (PDEIII) ($IC_{50} = 0.9$ vs 36 nM) this activity did not correlate with the potency of inhibition of megakaryocyte development. Furthermore, other PDEIII inhibitors of widely differing potency were shown to have negligible effects on megakaryocytopoiesis.

6 Taken together our results demonstrate that anagrelide and BCH24426 target a cellular event involved specifically in the megakaryocyte differentiation programme, which is independent of PDEIII inhibition.

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Keywords: Anagrelide; metabolites; megakaryocyte; haematopoiesis; phosphodiesterase; myeloproliferative disorders

Abbreviations: ANOVA, analysis of variance; BCH24426, 6,7-dichloro-3-hydroxy-1,5 dihydro-imidazo[2,1-b]quinazolin-2-one; CD, cluster differentiation antigen number; DMSO, dimethyl sulfoxide; EPO, erythropoietin; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage colony-stimulating factor; GpA, glycoprotein A; IBMX, isobutyl-1-methylxanthine; IMDM, Iscove's-modified Dulbecco's medium; MK, megakaryocyte; PBS, phosphate-buffered saline; PDEIII, phosphodiesterase type III; RL603, 2-amino-5,6-dichloro-3,4,-dihydroquinazoline; SDF-1 α , stromal cell-derived factor-1 α ; TPO, thrombopoietin

Introduction

Anagrelide is an imidazoquinazoline derivative that was initially developed as an inhibitor of platelet aggregation (Fleming & Buyniski, 1979). However, when first tested in humans it showed profound thrombocytopenic effects (Abe *et al.*, 1984). This unexpected action prompted evaluation of the possible clinical utility of this property, which ultimately demonstrated the usefulness of the drug for the treatment of thrombocytosis in patients with chronic myeloproliferative disorders (reviewed in Pescatore & Lindley, 2000). Initial studies in humans indicated that anagrelide does not sig-

nificantly affect platelet-survival time (Abe *et al.*, 1984) nor that it acts by inducing haematopoietic progenitor cell toxicity (Silverstein *et al.*, 1988). Later on, *in vitro* studies of human megakaryocytopoiesis suggested that, *in vivo*, the thrombocytopenic activity of this compound results primarily from an inhibitory effect on the postmitotic phase of megakaryocyte (MK) maturation (Mazur *et al.*, 1992). However, notwithstanding its clinical success and these pioneering cellular studies, unravelling the primary mechanism of action by which anagrelide reduces platelet count has remained elusive (Hong & Erusalimsky, 2002).

Anagrelide is extensively metabolized by the liver (Pescatore & Lindley, 2000). Early studies identified

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2-amino-5,6-dichloro-3,4-dihydroquinazoline (RL603) as a major metabolite of the drug. Lane *et al.* (2001) investigated the activity of RL603 in MK cultures and also in the mouse. Their work suggested that this compound might be endowed with platelet-lowering activity, arising from inhibitory effects on MK maturation and migration. However, *in vitro* studies in our laboratories did not support this contention (Erusalimsky *et al.*, 2002). A second major metabolite of anagrelide, 6,7-dichloro-3-hydroxy-1,5-dihydro-imidazo[2,1-b]quinazolin-2-one, has recently been identified (Figure 1). Known as BCH24426, the activity of this metabolite has not been previously investigated.

To date the only known primary target of anagrelide is a type III phosphodiesterase (PDEIII) found in platelets and the myocardium (Gillespie, 1988; Beavo, 1995). While it would seem improbable that inhibition of this enzyme could account for the effects of anagrelide on MK development, this possibility has not been formally investigated.

To assess the potential role that the metabolites of anagrelide may play in the antithrombopoietic action of the parent compound in this study we have compared the activities of anagrelide, RL603 and BCH24426 for their effects on thrombopoietin (TPO)-induced haematopoietic progenitor CD34⁺ cell expansion, as well as MK development and migration in culture. Furthermore, we have compared these compounds with other known PDEIII inhibitors in order to evaluate whether PDEIII inhibition could explain the anti-thrombopoietic activity of anagrelide.

Methods

Chemicals

Anagrelide hydrochloride monohydrate (Batch No CML-227/01-RS6, >99% purity) was obtained from Cambridge Major Laboratories Inc., Germantown, WI, U.S.A. BCH24426 (Batch No MSC114/15, >94% purity) was synthesized by Tocris Cookson, Bristol, U.K. RL603 was obtained from Ultrafine, Manchester, U.K. (Batch No 116-44-1, 98% purity) or from York Bioanalytical Solutions, Upper Poppleton, U.K. (Batch No 130-24-1, purity ~100%). Cilostazol was obtained from Sigma-Aldrich, Poole, U.K. Trequinsin hydrochloride, milrinone and cilostamide were purchased from Calbiochem, Nottingham, U.K. Isobutyl-1-methylxanthine (IBMX) was from Sigma, Poole, U.K. Trequinsin hydrochloride was dissolved in water. Stocks solutions of RL603 were made in dimethyl sulfoxide (DMSO) or in acidic (pH 5.0) phosphate-buffered saline (PBS). All other test compounds were dissolved in DMSO. Stock solutions (10 mM) were stored at -20°C in small aliquots and when required, diluted in culture medium immediately before addition to cell suspensions.

Cells

Umbilical cord blood was obtained from normal donors undergoing scheduled caesarean births and who gave informed written consent in accordance to University College London Ethics Committee guidelines. CD34⁺ haematopoietic progenitor cells were isolated by magnetic immunoselection using the anti-CD34 monoclonal antibody QBEND/10 (Miltenyi Biotec Ltd, Surrey, U.K.) as previously described (Mathur *et al.*,

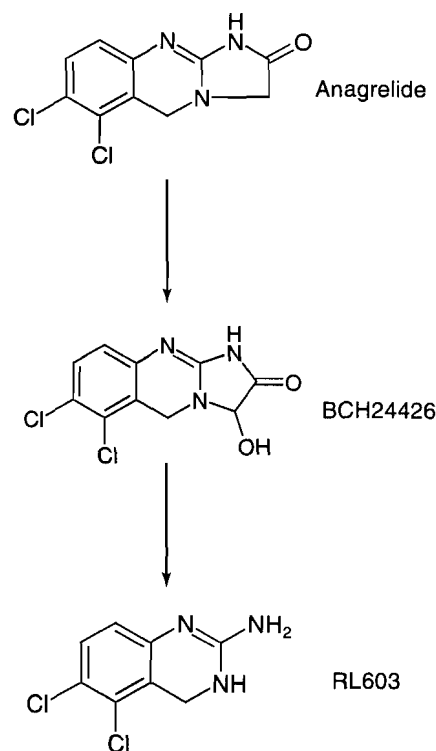


Figure 1 Metabolic pathway of anagrelide in man. Determined by mass spectrometric analysis of urine from volunteers given a single oral 1 mg dose of [¹⁴C]-labelled anagrelide.

2004). Cell purity was checked by flow cytometry and ranged between 92 and 98%.

Cell culture and treatments with test compounds

To examine the effect of test compounds on the expansion of CD34⁺ cells resulting from TPO stimulation, these cells were grown under conditions which support the proliferation of MK progenitors in serum-free medium (Lam *et al.*, 2001; van den Oudenrijn *et al.*, 2001). Freshly isolated cells were cultured with 40 ng ml⁻¹ recombinant human TPO (R&D Systems, Abingdon, U.K.) in Stemspan™ medium (Stem Cell Technologies, London, U.K.) which consisted of Iscove's-modified Dulbecco's medium (IMDM) supplemented with 1% bovine serum albumin, 10 µg ml⁻¹ recombinant human insulin, 200 µg ml⁻¹ iron-saturated transferrin, 0.1 mM 2-mercaptoethanol and 2 mM glutamine. Cells were seeded onto flat bottom 96-well microtitre plates (Falcon) at an initial density of 1.5 × 10⁵ cells ml⁻¹ in a volume of 100 µl and incubated at 37°C in a humidified incubator under 5% CO₂/95% air. At 1 day after the initial seeding, test compounds or vehicle were added at the indicated doses in a volume of 10 µl and the cells were cultured for a further 5 days. At the end of the culture period the relative number of viable cells was determined using a tetrazolium salt-based colourimetric assay (XTT, Cell proliferation kit II, Roche Diagnostics GmbH, Mannheim, Germany) as described by the manufacturers.

To promote terminal differentiation, unless otherwise indicated, freshly isolated CD34⁺ cells were cultured in

standard differentiation medium consisting of IMDM (Sigma) supplemented with 10% human umbilical cord blood plasma, 0.2% bovine serum albumin, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (Gibco BRL), minimal essential medium vitamins (Gibco BRL), 0.1 mM 2-mercaptoethanol, 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin and specified haematopoietic growth factors. In some experiments cells were cultured in the above-described Stemspan™ serum-free medium. Cells were grown for 12 days at 37°C in a humidified incubator under 5% CO₂/95% air. The final cell density of the differentiated cultures was determined using a Sysmex CDA-500 Particle Analyser fitted with a 5–20 µm probe. Treatments with test compounds under differentiation conditions were carried out according to two alternative schedules. Schedule A was used to evaluate the activity of test compounds on the growth and differentiation of developing MKs. In this case cells were plated at a density of 2.0×10^5 cells ml⁻¹ in medium containing 40 ng ml⁻¹ TPO. After 4 days of culture, cells were counted and then diluted (~3-fold) to 1.5×10^5 cells ml⁻¹ by addition of fresh medium supplemented with 10 ng ml⁻¹ TPO. Following dilution, cell aliquots of 1 ml were replated into 24-well plates and test compounds or vehicle were added at the appropriate doses in a volume of 20 µl. Cells were then left to grow undisturbed until the end of the 12-day culture period. Schedule B was used to compare the activity of test compounds against the differentiation of CD34⁺ cells into different lineages as follows: Cell aliquots of 1 ml were seeded onto 24-well plates at a density of 1.5×10^5 cells ml⁻¹ with medium containing either 40 ng ml⁻¹ TPO, 8 U ml⁻¹ recombinant human erythropoietin (EPO, Roche Diagnostics GmbH) or 20 ng ml⁻¹ recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems). After an overnight incubation, drugs or vehicle were added as described above. Subsequently, the cells were left to grow undisturbed until the end of the 12-day culture period.

Flow cytometric analysis of cell differentiation

Phenotypic differentiation was monitored by flow cytometry using the following fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies: Y2/51 (DAKO, U.K.), which detects the megakaryocytic lineage-specific marker CD61; CLB-409 (Cymbus Biotechnology, Hants, U.K.), which recognizes the erythroid differentiation marker glycophorin A (GpA) and MΦP9 (Becton Dickinson, U.K.), which detect the antigen CD14 on myelomonocytic cells. Cells were stained and analysed as previously described (Bobik *et al.*, 1998; Mathur *et al.*, 2004). The boundary between antigen-positive and -negative cells was established according to the fluorescence distribution of cells stained with an isotype-matched control antibody. The number of differentiated cells was estimated by multiplying the total number of cells in the culture by the percentage of antigen-positive cells. Relative cell size was determined by flow cytometry from the mean forward scatter signal of the cell population. Determination of DNA ploidy distributions in megakaryocytic cultures was carried out by double labelling with FITC-conjugated Y2/51 and propidium iodide (Sigma) as previously described (Mathur *et al.*, 2004).

Transmigration assay

Transmigration experiments were carried out using a bicompartamental system (Costar, U.K.) consisting of a Transwell® chamber (5 µm membrane pore size) inserted onto the well of a 24-well tissue culture plate essentially as we have previously described (Mathur *et al.*, 2001). Experiments were performed using unfractionated terminally differentiated MK cultures ($1.0\text{--}1.5 \times 10^6$ cells ml⁻¹) that had been previously grown in standard differentiation medium containing 40 ng ml⁻¹ TPO as described above. The proportion of CD61⁺ cells in these cultures ranged between 65 and 80%. Cell suspensions were used in their own conditioned medium without further processing. Aliquots of the cell suspension (0.1 ml) were placed onto the Transwell and test compounds, or an equivalent amount of vehicle, were added to give a final concentration of 1 µM. The lower compartment was filled with fresh 0.6 ml standard differentiation medium with or without 150 ng ml⁻¹ stromal cell-derived factor 1α (SDF-1α, R&D Systems). Plates were placed in an incubator at 37°C under 5% CO₂ for 4 h. At the end of this period the Transwell insert was removed and migrated cells were collected from the bottom well. Both the bottom well and the external surface of the insert were rinsed with 1 ml PBS containing 2 mM EDTA to ensure that all cells were collected. SDF-1α-induced MK migration was quantified by flow cytometry following staining of the collected cells with FITC-conjugated Y2/51 monoclonal antibody as described above. Each sample was resuspended in a final volume of 0.4 ml and cells were counted twice using the flow cytometer time parameter, which was set to acquire events for 30 s. Analysis gates were established to assess the migration of CD61-positive and -negative cells. Regular checks were made to ensure that the volume of cells processed by the instrument during the 30-s acquisition period remained constant.

Data processing and statistical analysis

Each experiment was carried out in 2–3 replicates with cells derived from the same donor and the results were averaged. Experiments were repeated at least three times using each time CD34⁺ cells from a different donor. To account for variations in the differentiation responses between different batches of CD34⁺ cells, results were normalized to percentages of control (no drug added) and then averaged. Statistical analysis was carried out using SPSS software (release 12, SPSS Inc., Chicago, IL, U.S.A.). A two-tailed Student's *t*-test was used to establish levels of significance for differences between two values. For comparing dose-responses levels of significance were determined by two-way analysis of variance (ANOVA). Differences resulting in $P < 0.05$ were considered significant. IC₅₀ values were calculated using Prism software (release 3.0, GraphPad Software Inc., San Diego, CA, U.S.A.).

Results

Activity of anagrelide and its metabolites against the early expansion of CD34⁺ cell cultures stimulated by TPO

Figure 2 shows the dose-responses for the effects of anagrelide, RL603 and BCH24426 on the early expansion of

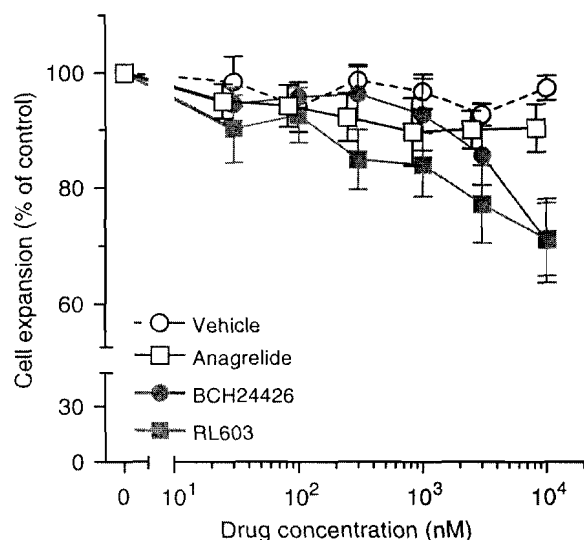


Figure 2 Dose-responses for the effects of anagrelide and its metabolites on TPO-induced CD34⁺ cell expansion. CD34⁺ cells were cultured for 6 days in Stemspan[™] serum-free expansion medium supplemented with TPO. The indicated compounds or an equivalent amount of vehicle (DMSO) were added after 24 h. Cell expansion was determined by the XTT assay and is expressed relative to untreated samples grown in parallel. Results represent the mean \pm s.e.m. of three to five experiments.

CD34⁺ cell cultures grown with TPO in serum-free medium. Under these experimental conditions (6 days of culture), control cells underwent 6.9 ± 1.9 -fold expansion ($n=5$). Each of the test compounds had weak inhibitory activity against this process (5–15% inhibition at $1 \mu\text{M}$, reaching 10–30% at $10 \mu\text{M}$). Of the three compounds anagrelide appeared to be the weakest (ANOVA, $P=0.06$ vs vehicle), whereas RL603 and BCH24426 showed stronger comparable activity (ANOVA, $P<0.01$ for each treatment vs vehicle). However, due to the variability in the responses only the latter demonstrated a significant effect of dose (ANOVA, $P=0.02$ vs vehicle) and this was accounted exclusively by a reduction in the number of viable cells at the highest concentration tested ($\sim 30\%$ inhibition at $10 \mu\text{M}$ BCH24426, $P=0.004$ vs vehicle by *t*-test). Furthermore, comparison between the dose-responses of anagrelide and BCH24426 showed the two compounds to be indistinguishable (ANOVA, $P=0.12$).

Differential effects of anagrelide metabolites against megakaryocytopoiesis

To compare the effects of anagrelide and its metabolites on the *ex vivo* production of MKs, CD34⁺ cells were grown in differentiation medium supplemented with TPO and test compounds were added after 1 or 4 days of culture (schedules B and A, respectively). Preliminary experiments demonstrated that there was no substantial difference in the final effect of the test compounds using these alternative schedules (data not shown). However, because schedule A allows for the expansion of the MK progenitors before they are exposed to the test compounds, a much large number of samples can be tested in parallel under these conditions. Hence schedule A was used in most of the subsequent experiments in which MK

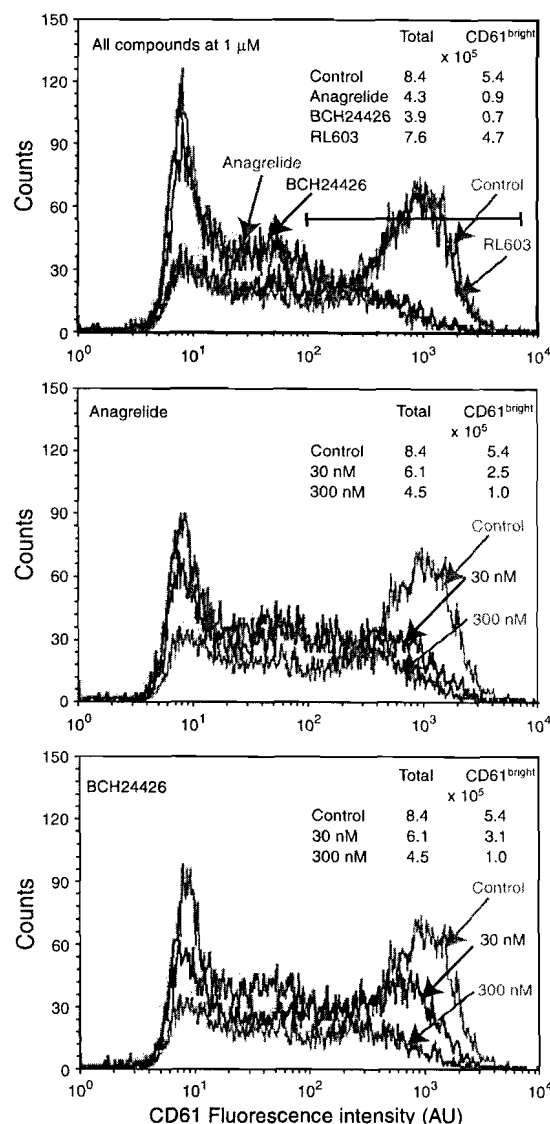


Figure 3 Flow cytometric histograms of CD61 expression in cultured MKs treated with anagrelide or its metabolites. CD34⁺ cells were cultured for 12 days in differentiation medium supplemented with TPO in the presence or absence of the indicated compounds as described in schedule A under Methods and then analysed by flow cytometry. The marker encompasses the CD61^{bright}-positive cells. The inset tables show both the total number of cells and the number of CD61^{bright}-positive cells in the respective cultures.

development was examined. Figure 3 shows representative flow cytometric profiles of CD61 expression (a measure of differentiation) after 12 days of culture. In control cultures, the majority of cells expressed very high relative levels of this megakaryocytic differentiation marker (~ 100 -fold above those of the negative fraction). Addition of anagrelide or BCH24426 reduced the fraction of CD61^{bright} cells in a dose-dependent manner, with a maximal effect observed at $\sim 1 \mu\text{M}$ (Figure 3 and data not shown). In contrast, flow cytometric profiles of RL603-treated cultures were virtually indistinguishable from those of control cultures (Figure 3a and data not shown). As shown on Table 1 at a concentration of $1 \mu\text{M}$

Table 1 Effects of anagrelide and its metabolites on the growth characteristics of MK cultures

Treatment	Cell density ^a (%)	Relative cell size ^b (%)	CD61 expression	
			CD61 ^{bright} cell fraction ^a (%)	Median CD61 fluorescence ^b (%)
Vehicle	95.8 ± 2.2	105.5 ± 3.0	99.1 ± 1.6	98.5 ± 3.4
Anagrelide	48.0 ± 4.0**	80.4 ± 4.5**	41.9 ± 6.6**	51.3 ± 11.9**
BCH24426	45.8 ± 3.9**	78.7 ± 5.3**	34.5 ± 4.4**	54.4 ± 14.7*
RL603	93.9 ± 7.2	103.1 ± 4.1	94.2 ± 2.2	90.2 ± 13.3

CD34⁺ cells were cultured in differentiation medium supplemented with TPO in the presence or absence of the indicated compounds (1.0 μ M) or an equivalent amount of vehicle (0.01% DMSO) as described in Figure 3 and then analysed by flow cytometry. Results are expressed in percentages relative to untreated cells grown in parallel. Values represent the mean \pm s.e.m. of four experiments. * P < 0.05; ** P < 0.01 vs vehicle.

^aValues correspond to the total cell population.

^bValues correspond to the CD61^{bright} cell fraction.

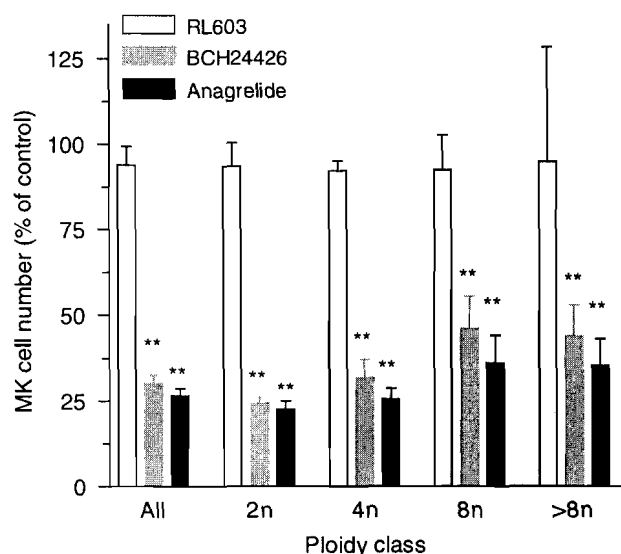


Figure 4 Effects of anagrelide and its metabolites on the ploidy distribution of cultured MKs. CD34⁺ cells were cultured with the indicated compounds (1.0 μ M) as described in Figure 3. Results show the total number of CD61^{bright} cells and the number in each ploidy class relative to untreated samples grown in parallel. Values represent the mean \pm s.e.m. of three or four experiments. ** P < 0.01 vs vehicle.

anagrelide and BCH24426 reduced the fraction of positive cells by an average of 60–65%. In addition, these compounds reduced significantly the median fluorescence intensity of the CD61^{bright} subpopulation (a measure of the relative level of antigen expression per cell), the average cell size of the whole culture (the latter is a function of both cytoplasmic maturation and DNA content) and its final cell density. In contrast, RL603 had no significant effect on any of these variables (Table 1). Furthermore, flow cytometric analysis of ploidy distributions showed that anagrelide and BCH24426 reduced the proportion of MKs in each ploidy class to similar extents whereas RL603 again showed no significant effect (Figure 4).

Figure 5 shows the dose-responses for the overall activity of anagrelide and its metabolites against *in vitro* megakaryocytopoiesis as assessed by evaluating their effects on the total number of MKs (CD61^{bright} cells) produced in the culture. These results show that anagrelide and BCH24426 inhibit

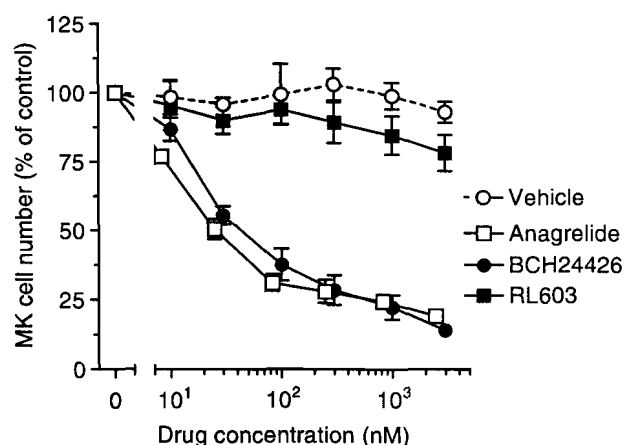


Figure 5 Dose-responses for the effects of anagrelide and its metabolites on TPO-induced MK development. CD34⁺ cells were cultured with the indicated compounds as described in Figure 3. Results show the total number of CD61^{bright} cells relative to untreated samples grown in parallel. Values represent the mean \pm s.e.m. of eight experiments.

MK development with high efficacy and very similar potencies (mean IC₅₀ \pm s.e.m. = 26 \pm 4 and 44 \pm 6 nM, respectively; ANOVA, P = 0.8 between compounds). In contrast, in the same concentration range RL603 showed only weak activity and no significant effect of dose (ANOVA, P = 0.5 compared to vehicle); hence in this case an IC₅₀ value could not be established.

The poor effect of RL603 on MK development compared to the strong inhibition of this process by anagrelide or BCH24426 seen in the above described experiments was reproduced when cultures were grown for 12 days in serum-free medium, when RL603 was prepared in acidic PBS or when material from an alternative supplier was tested (data not shown).

Selective inhibition of megakaryocytopoiesis by anagrelide and BCH24426

To assess whether the inhibitory activities of anagrelide and BCH24426 in these cultures were selective for the megakaryocytic lineage, we examined the effects of these compounds on the growth of the nonmegakaryocytic cells (CD61[−]). In 12-day control cultures, these cells represent 20–35% of the total

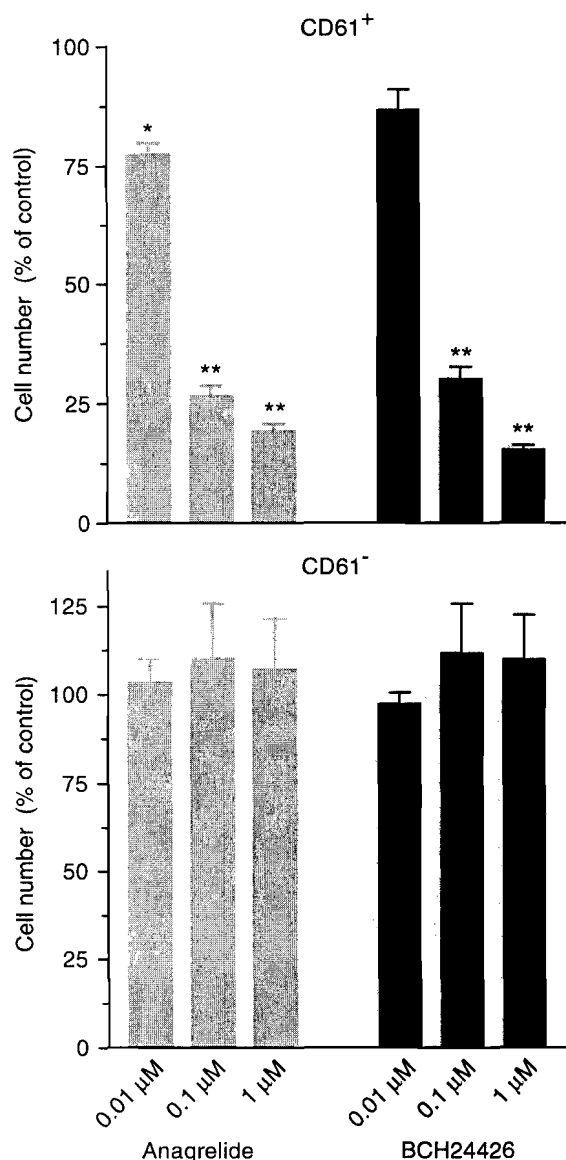


Figure 6 Effect of anagrelide and BCH24426 on the growth on nonmegakaryocytic cells. CD34⁺ cells were cultured with the indicated compounds as described in Figure 3. Results show the total number of CD61⁻ and CD61⁺ cells relative to untreated samples grown in parallel. Values represent the mean \pm s.e.m. of four experiments. * P < 0.05; ** P < 0.01 vs vehicle.

population (Figure 3). As depicted in Figure 6, in sharp contrast to the reduction in the final number of cells expressing CD61, neither anagrelide nor BCH24426 inhibited the growth of nonmegakaryocytic cells.

To obtain further evidence for the selectivity of these compounds their effects on the differentiation of CD34 cells into other haematopoietic lineages was also tested. As shown in Figure 7, neither anagrelide nor its metabolites had a significant effect on erythroid or myelomonocytic differentiation induced by EPO or GM-CSF. In contrast, in the same experiments, both anagrelide and BCH24426 had a strong inhibitory effect on megakaryocytic differentiation induced by TPO.

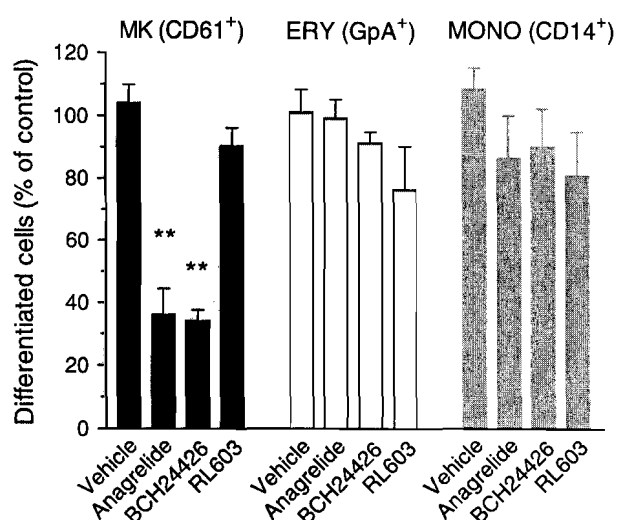


Figure 7 Comparison between anagrelide and its metabolites for their effects on haematopoietic lineage differentiation. CD34⁺ cells were cultured for 12 days in differentiation medium supplemented with TPO, EPO or GM-CSF in the presence or absence of the indicated compounds (1.0 μ M) or an equivalent amount of vehicle (0.01% DMSO) as described in schedule B under Methods. Results show the total number of megakaryocytic (MK, CD61⁺), erythroid (ERY, GpA⁺) and myelomonocytic (MONO, CD14⁺) cells relative to untreated samples grown in parallel. Values represent the mean \pm s.e.m. of four experiments. ** P < 0.01 vs vehicle.

Lack of activity of anagrelide and its metabolites against SDF-1 α -stimulated MK migration

Figure 8 shows the results of a representative experiment in which anagrelide and its metabolites were tested for their effects on the migration of cells derived from CD34⁺ cell cultures. Both CD61⁺ megakaryocytic cells and CD61⁻ nonmegakaryocytic cells exhibited a strong migratory response to the chemotactic factor SDF-1 α . However, neither anagrelide nor its metabolites showed a significant effect on the migratory response. It has been suggested that preparation of RL603 in acidic aqueous buffers increases its solubility and hence its activity (Raffi & Lane, 2002). However, as shown in Figure 8, when RL603 was prepared in this manner it still failed to inhibit SDF-1 α -induced migration.

In order to confirm these findings experiments were repeated several times using cells from different donors. As shown in Table 2, on average all the compounds caused a small inhibition of SDF-1 α -dependent MK migration. However, none of the effects reached statistical significance.

Evidence that phosphodiesterase inhibition is not related to the antimegakaryocytopoietic activity of anagrelide and BCH24426

As shown in Table 3, consistent with previous reports (Gillespie, 1988) anagrelide was found to inhibit PDEIII *in vitro*. Furthermore, BCH24426 was found to be 40-fold more potent than anagrelide against this activity. These findings contrasted with the fact that both compounds had similar potencies when tested for their effects on megakaryocytopoiesis (Figure 5) and thus suggest that the mechanism by which they abrogate this process is unrelated to PDEIII inhibition.

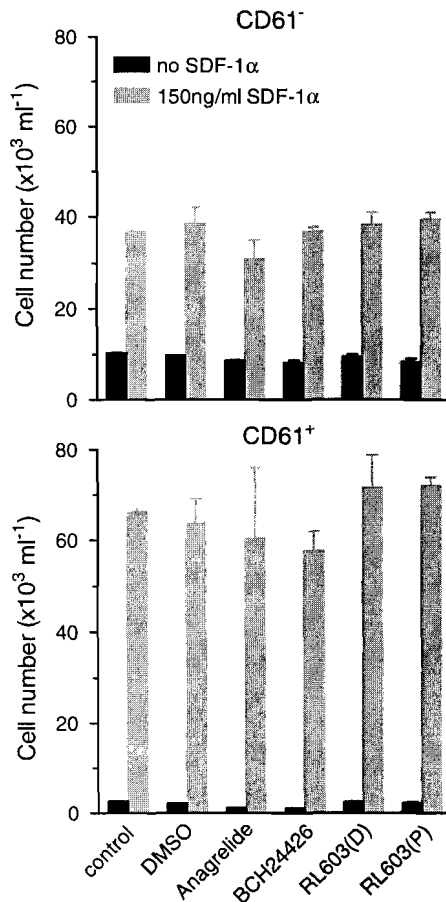


Figure 8 Effects of anagrelide and its metabolites on the transmigration of CD61⁺ and CD61⁻ cells. Transmigration experiments were carried out in the presence or absence of the indicated compounds (1.0 μ M) or 0.01% DMSO as described under Methods. Results represent the mean \pm s.e.m. number of migrated cells for three determinations in one representative experiment. RL603 was dissolved in DMSO (D) or in acidic PBS (P).

In order to substantiate this contention, further experiments were carried out with a number of commercially available PDEIII inhibitors. As summarized in Table 3 while both anagrelide and BCH24426 showed strong and significant inhibitory effects against MK development, apart from trequinsin (*t*-test, $P=0.04$ vs untreated cells), none of the other PDEIII inhibitors showed a statistically significant effect against this process. In the case of trequinsin, however, the overall effect was small, even though the compound was used at a concentration equivalent to 3000-fold its IC_{50} for PDEIII.

Discussion

In these investigations we have examined the activity of two major metabolites of anagrelide, RL603 and BCH24426, against several *in vitro* parameters related to the process of megakaryocytopoiesis and platelet production *in vivo*. These parameters included the early expansion of undifferentiated MK progenitors, the degree of MK maturation (relative cell size, ploidy and level of CD61 expression), the overall number

Table 2 Effects of anagrelide and its metabolites on MK migration

Treatment	SDF-1 α -dependent MK migration (%)	P*
DMSO, 0.01%, $n=5$	98.0 \pm 7.9	
Anagrelide, $n=5$	83.3 \pm 10.3	0.29
BCH24426, $n=5$	74.2 \pm 8.9	0.08
RL603 (D) ^a , $n=5$	83.6 \pm 8.3	0.25
RL603 (P) ^a , $n=3$	80.4 \pm 18.2	0.29

Transmigration experiments were carried out as described in Figure 8. Results show the total number of CD61⁺ cells that migrated towards the SDF-1 α gradient, expressed as a percentage of cells migrating towards the gradient in the absence of test compound. Values represent the mean \pm s.e.m. of the indicated number experiments.

* P values were calculated by a *t*-test; RL603 (P) was compared to untreated cells and the remaining compound treatments were compared to DMSO.

^aThe letters in brackets denote whether RL603 was dissolved in DMSO (D) or acidic PBS (P).

Table 3 Comparison between PDEIII inhibitors for their activity against megakaryocytopoiesis

Treatment	PDEIII activity IC_{50} (nM)	MK cell number ^a (%)
DMSO, 0.01%	—	91.8 \pm 7.3
Trequinsin	0.3 ^b	83.5 \pm 5.3 [#]
BCH24426	0.9 ^c	27.5 \pm 3.9**
Cilostamide	27 ^b	86.3 \pm 7.1
Anagrelide	36 ^c	26.1 \pm 2.7**
Cilostazol	200 ^b	79.1 \pm 8.0
Milrinone	450 ^b	94.6 \pm 7.1
IBMX	3950 ^b	85.8 \pm 8.6

CD34⁺ cells were cultured in differentiation medium supplemented with TPO in the presence or absence of the indicated compounds (1.0 μ M) or an equivalent amount of DMSO as described in schedule A under Methods.

^aThe number of MKs in the cultures is shown in percentages relative to untreated cells; values represent the mean \pm s.e.m. of three experiments.

^b IC_{50} values for trequinsin (Ruppert & Weithmann, 1982), Cilostamide (Sudo *et al.*, 2000), cilostazol (Sudo *et al.*, 2000), milrinone (Sudo *et al.*, 2000) and IBMX (Tang *et al.*, 1994) were obtained from the literature.

^cPDEIII activity was measured by assessing the production of 5'-AMP from cAMP using enzyme isolated from human platelets.

[#] $P < 0.05$ vs untreated cells; ** $P < 0.01$ vs DMSO.

of CD61-expressing cells and the migration of MKs stimulated by SDF-1 α . Our results demonstrate that BCH24426 is a potent inhibitor of megakaryocytopoiesis, with virtually identical effects to those observed with anagrelide, in terms of potency, efficacy, mode of action and specificity. These conclusions are based on the following findings: Firstly, anagrelide and BCH24426 affected the same MK differentiation parameters and to similar extents (Table 1, Figures 3 and 4). Secondly, both drugs showed similar IC_{50} values for their overall effect on the generation of differentiated MKs (Figure 5). Thirdly, in the 0.1–1 μ M concentration range, that is, at concentrations that effectively inhibited MK development, neither compound showed a substantial effect against the TPO-induced early expansion of progenitor cells (Figure 2), against the growth of nonmegakaryocytic cells (Figure 6), against the differentiation of haematopoietic progenitor cells

into other lineages (Figure 7) or against MK migration (Figure 8). Thus, taken together these findings suggest that both anagrelide and BCH24426 act selectively on the growth and maturation of committed MK precursors.

Cell culture studies carried out by another laboratory had previously suggested that RL603 is 50 times more potent than anagrelide as an inhibitor of MK maturation and that this compound also inhibits MK migration (Lane *et al.*, 2001). In preliminary studies (Erusalimsky *et al.*, 2002), which we have now extended by the present work, we could not confirm those claims. Indeed, while we found some evidence that RL603 has antimegakaryocytopoietic activity, the overall inhibition was small, even at relatively high doses (Figure 5). Furthermore, we have not found any evidence that RL603 inhibits MK migration to a significant extent (Figure 8 and Table 2). Rafii & Lane (2002) have previously indicated that in order to increase the solubility of RL603, the compound ought to be prepared in acidic aqueous media and/or that the presence of DMSO may interfere with the sensitivity of MK development bioassays. However, in the present work we could not corroborate those claims. Indeed, in our hands the activity of RL603 did not improve when the drug was prepared in acidic aqueous media and when there were no traces of DMSO in the assay. Another possibility is that differences in experimental culture conditions accounted for these contradictory findings. Rafii & Lane (2002) carried out their *in vitro* studies in plasma-free medium. However, in our studies the use of plasma-free or plasma-containing media did not affect the activity of RL603. Consistent with these *in vitro* findings, *in vivo* studies conducted by one of the authors failed to show any effect of RL603 on platelet counts when administered to rats or mice (Franklin, unpublished data). Thus, an explanation for these apparently conflicting data on the activity of RL603 would require further experimental work.

A salient finding of the present study is that anagrelide and BCH24426 inhibited MK development at low nanomolar concentrations (Figure 5). In the case of anagrelide these doses are well within the range of therapeutic concentrations reached in the human circulation. For instance, after administration of a typical 1 mg oral dose to patients with essential thrombocythaemia the mean maximum plasma concentration was $6.2 \text{ ng ml}^{-1} = 24 \text{ nM}$ (data on file at Shire Pharmaceuticals). In the case of BCH24426 the corresponding mean maximum plasma concentration was $8.7 \text{ ng ml}^{-1} = 32 \text{ nM}$, which again lies within the range observed to be effective in the current *in vitro* studies.

Previous findings have suggested that anagrelide acts primarily on the postmitotic phase of MK maturation (Mazur *et al.*, 1992; Solberg *et al.*, 1997). More recently work by another laboratory has shown that *in vivo* anagrelide also

reduces MK cell numbers (Tomer, 2002). Consistent with this finding our results in liquid cultures show that nanomolar concentrations of anagrelide and BCH24426 not only affect the degree of maturation (Figure 3 and Table 1) but also caused a dose-dependent decline in the overall number of MKs (Figure 5). This reduction in MK cell number could not be attributed to indiscriminate cytotoxic effects, as the compounds did not reduce the number of nonmegakaryocytic cells in cultures grown with TPO (Figure 6), nor did they significantly affect the number of erythroid or myelomonocytic cells in cultures grown with EPO and GM-CSF, respectively (Figure 7). Furthermore, the lack of significant interference with the expansion of early MK progenitors at concentrations, which effectively inhibited overall MK development, that is, in the $0.1\text{--}1.0 \mu\text{M}$ range (compare Figure 2 with 5), suggest that anagrelide and BCH24426 inhibit proliferation at a late stage in the mitotic phase of megakaryocytopoiesis, that is, once a substantial degree of differentiation has taken place. This possibility is entirely consistent with the notion that there is a considerable degree of overlap between the proliferative and maturation phases of megakaryocytopoiesis (Long, 1993; Hong & Erusalimsky, 2002).

It could be argued that the reduction in MK numbers is due to the active compounds having a selective toxic effect on the developing MK subpopulation. However, this possibility is unlikely because effective concentrations of anagrelide did not increase the basal level of cell death in these cultures (Hong & Erusalimsky, manuscript in preparation).

Anagrelide inhibits PDEIII found in platelets and as a result raises cAMP levels in these cells (Gillespie, 1988; Seiler *et al.*, 1987). This action may explain its inhibitory effect on platelet aggregation. Although after *in vivo* administration of doses which are effective in reducing blood platelets, this activity is very short lived, the role of PDEIII inhibition in the thrombocytopenic action of the drug has never been entirely discounted. BCH24426 like anagrelide inhibits PDEIII *in vitro*, the former being 40 times more potent. These wide differences in potency with regards to PDEIII inhibition but not with regards to megakaryocytopoiesis, and the fact that a number of other known phosphodiesterase inhibitors did not have any effect on the latter (Table 3) strongly suggests that PDEIII inhibition is not the mechanism by which anagrelide inhibits platelet production. In conclusion, our data indicate that anagrelide and BCH24426 target a cellular event involved specifically in the megakaryocytic differentiation programme. These findings suggest that *in vivo* BCH24426 could also play a role in the platelet-lowering action of the parental compound.

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